Environmental Research 164 (2018) 647–654

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Combined effects of warming and acidification on accumulation and elimination dynamics of paralytic shellfish toxins in mussels *Mytilus galloprovincialis*

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ARTICLE INFO

Keywords:
Harmful algal blooms
Climate change
Seafood safety
Saxitoxins
Gymnodinium catenatum

ABSTRACT

Harmful algal blooms (HAB) have been increasing in frequency and intensity most likely due to changes on global conditions, which constitute a significant threat to wild shellfish and its commercial farming. This study evaluated the impact of increasing seawater temperature and acidification on the accumulation/elimination dynamics of HAB-toxins in shellfish. *Mytilus galloprovincialis* were acclimated to four environmental conditions simulating different climate change scenarios: *i*) current conditions, *ii*) warming, *iii*) acidification and *iv*) interaction of warming with acidification. Once acclimated, mussels were exposed to the paralytic shellfish toxins (PSTs) producing dinoflagellate *Gymnodinium catenatum* for 5 days and to non-toxic diet during the subsequent 10 days. High toxicity levels (1493 μg STX eq. kg^{-1}) exceeding the safety limits were determined under current conditions at the end of the uptake period. Significantly lower PSP toxicity levels were registered for warming- and acidification-acclimated mussels (661 and 761 μg STX eq. kg^{-1}). The combined effect of both warming and acidification resulted in PSP toxicity values slightly higher (856 μg STX eq. kg^{-1}). A rapid decrease of toxicity was observed in mussels at the current conditions after shifting to a non-toxic diet, which was not noticed under the predicted climate change scenarios. Variability of each PST analogue, measured throughout the experiment, highlighted different mechanisms are associated with changes of each environmental factor, although both resulting in lower toxicity. Warming-acclimated mussels showed lower accumulation/elimination rates, while acidification-acclimated mussels showed higher capability to accumulate toxins, but also a higher elimination rate preventing high toxicity levels. As different mechanisms are triggered by warming and acidification, their combined effect not leads to a synergism of their individual effects. The present work is the first assessing the combined effect of climate change drivers on accumulation/elimination of PSTs, in mussels, indicating that warming and acidification may lead to lower toxicity values but longer toxic episodes. PSTs are responsible for the food poisoning syndrome, paralytic shellfish poisoning (PSP) in humans. This study can be considered as the first step to build models for predicting shellfish toxicity under climate change scenarios.

1. Introduction

Most coastal areas are highly productive and simultaneously vulnerable ecosystems that have been continuously affected by multiple man-made pressures. The impacts of climate change are likely to deteriorate environmental conditions and worsen ecological problems in these sensitive coastal ecosystems, where bivalves play a significant role. Bivalves are of high ecological importance, increasing local biomass, promoting biodiversity, and being responsible for some ecosystem engineering processes (Sousa et al., 2009; Fernández-Reiriz et al., 2012). In addition, these molluscs are an import source of protein for human consumption, representing valuable resources for coastal populations.

Warming, sea level rise, changes on circulation and salinity patterns, nutrient and sediment regimes, and acidification have been pointed out as the major climate change drivers affecting coastal areas

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https://doi.org/10.1016/j.envres.2018.03.045
Received 6 February 2018; Received in revised form 29 March 2018; Accepted 31 March 2018
Available online 06 April 2018
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(Filgueira et al., 2016). The Intergovernmental Panel on Climate Change (IPCC) indicates that, not only, the water temperature increased in the last century, but also acidification occurred, with a decrease of the water pH by almost 0.6 pH units (IPCC, 2013). These trends are projected to worsen, with temperatures increasing over 4.5 °C and pH decreasing from 0.3 to 0.5 pH units by 2100 in the worst case scenarios (IPCC, 2013). While IPCC values are projected for the end of the century, global ocean conditions, coastal areas and intertidal organisms, such as shellfish, are nowadays being frequently challenged with abrupt changes of environmental conditions (Filgueira et al., 2016).

Thermal tolerance with changing temperatures has been the focus of several studies (Pörtner et al., 2007). The increase of temperature usually leads to an increase in feeding and metabolic rates, promoting growth and reproduction when values are within the species thermal optimum range and in the presence of sufficient food and oxygen (Filgueira et al., 2016). However, when these conditions are not met, the increase in temperature may negatively affect shellfish. Changes in mussels behaviour, immune response, standard metabolic rate, reduction of energy available for the somatic metabolism, and negative impacts on growth rates have been associated with increasing temperature (Anestis et al., 2007; Coppola et al., 2017; Múgica et al., 2015). Increase of temperature may also promote the uptake, accumulation and metabolization of some pollutants, like metals (*e.g.* Ni and Cd) and radionuclides, as well as persistent organic pollutants (POPs) and methylmercury (Alava et al., 2017; Banni et al., 2014; Coppola et al., 2017; Dallas et al., 2016; Múgica et al., 2015; Sokolova and Lannig, 2008). Regarding marine biotoxins, namely paralytic shellfish poisoning toxins, temperature increase seems to affect their accumulation and elimination reducing both in oysters (Farrell et al., 2015) and increasing elimination surf clams (Bricelj et al., 2014).

In coastal upwelling zones, such as the Iberian Peninsula, the west coast of Americas, and the northwest and southwest coast of Africa, ocean acidification is anticipated to rise with the intensification of upwelling regimes (Bakun et al., 2015). Prolonged exposure to hypercapnia (acidified waters) may decrease the inter- and extra-cellular pH values and the consequent shell dissolution to compensate these acid-base disturbances (Parker et al., 2013). It also leads to changes in several physiological and metabolic processes like the reduction of thermal tolerance, decreased O_2 consumption, increased standard metabolic rates (SMR) and reduction of somatic growth rates (Michaelidis et al., 2005; Nikinmaa and Anttila, 2015; Parker et al., 2013).

The eastern boundary upwelling systems are among the most productive marine ecosystems. The nutrient pulses from deeper waters into the coastal photic zone, often resulting in phytoplankton blooms that are the basis of the marine food web. Among phytoplankton species blooming, some produce toxins as secondary metabolites, which are then accumulated in the biota, particularly by filter-feeding bivalves, and can become a public health concern. Recently, the largest and longest harmful *Pseudo-nitzschia* diatom bloom was observed along the west coast of the US associated with anomalously warming ocean conditions, being the bloom sustained by upwelled nutrients from the seasonal spring transition (Bond et al., 2015; McCabe et al., 2016). On the other hand, based on data from the last three decades, Pérez et al. (2010) pointed out a decline of coastal upwelling in the inner shelf of the Iberian Peninsula induced by sea surface warming. The upwelling decline and consequent less turbulent environment and persistence of stratified conditions may result in an increase in the frequency of toxic dinoflagellates blooms, such as *Dinophysis* spp. and *Gymnodinium catenatum,* profoundly impacting shellfisheries (Vidal et al., 2017). The bloom-forming dinoflagellates *Dinophysis* spp. and *G. catenatum* are linked to diarrhetic shellfish poisoning (DSP) and paralytic shellfish poisoning (PSP) outbreaks in humans, respectively. *G. catenatum* produces highly potent neurotoxins designated as paralytic shellfish toxins (PSTs). Its life-cycle includes a microreticulate resting cysts stage that facilitates their widespread distribution, either promoted by changes in natural conditions or by anthropogenic activities (Hallegraeff et al., 2012; Gobler et al., 2017). Harmful algal blooms (HAB), including *G. catenatum*-blooms, seem to be emerging and expanding in recent decades, which negatively affects the marine ecosystems and local economies due to increasing closures to shellfish harvesting (Gobler et al., 2017).

Research on the combined effects of warming seawater and acidification and exposure to harmful algal blooms on a bivalve production context is still scarce. Therefore, this study aims to assess the impact of both relevant climate change drivers *per se* and their combined effect on the dynamics of PSTs accumulation and elimination in *Mytilus galloprovincialis*.

2. Material and methods

2.1. Mussel collection and acclimation

One hundred and twenty immature Mediterranean mussels, *Mytilus galloprovincialis* (53.78 ± 6.22 mm), were harvested in Aveiro Lagoon in July 2016, when blooms of *G. catenatum* were not occurring. Upon collection, mussels were immediately shipped to IPMA facilities (Lisbon) in a thermally isolated container. Mussels were cleaned from macro-algae, barnacles or any other epibiont, and placed in four 150 L tank systems, subdivided into 6 replicates. Each tank systems was equipped with a protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal) to maintain seawater quality. Each tank was used to simulate a specific treatment: 1 – current conditions (Tr1: 19 °C; pH 8.0), 2 – warming (Tr2: 24 °C; pH 8.0), 3 – acidification (Tr3: 19 °C; pH 7.6) and 4 – warming and acidification (Tr4: 24 °C; pH 7.6) (Fig. 1). The seawater used was filtered (0.35 µm) and UV sterilised. Mussels were gradually adapted to the conditions of each treatment, by increasing 1 °C and decreasing 0.1 pH units per day. The animals were then acclimatised for 21 days, being fed with non-toxic and freeze-dried *Tetraselmis sp*. diet (Necton, Olhão, Portugal). Temperature and pH were automatically adjusted whenever needed. Water temperature was cooled through an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal) or heated by submerged digital heaters (200 W, V2Therm, TMC Iberia, Portugal). Water pH was measured through individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1 N, GHL, Germany), which monitored each tank every 2 s, and adjusted them whenever need, *via* submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or filtered air (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom).

Tanks were kept under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) salinity = 35.7 ± 0.4‰; iii) photoperiod = 12 L:12D (12 h light:12 h dark). Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Ammonia, nitrite and nitrate levels were daily checked by means of colorimetric tests (Tropic Marin, USA), and kept below detectable levels with daily water changes, except nitrates, which were kept below 2.0 mg L^{-1}. Seawater total alkalinity was also measured in every tank on a weekly basis, spectrophotometrically at 595 nm, following a protocol previously described elsewhere (Sarazin et al., 1999) and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters. A summary of seawater parameters is reported in Table A.1 and Fig. A.1 of Supplementary material.

2.2. Gymnodinium catenatum cultivation

G. catenatum strain IO-13-04, obtained from the algae culture collection at Lisbon University (ALISU), was isolated from a bloom in Espinho, NW Portuguese coast, in September 2005. Cells were mass

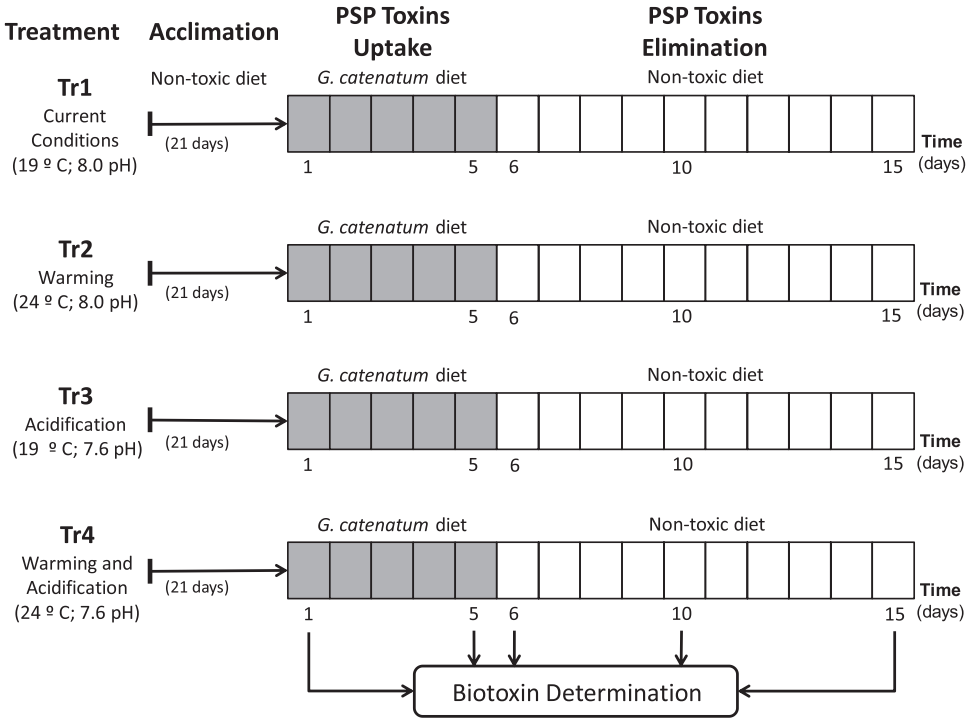

Fig. 1. Design of mussels feeding experiments with toxic dinoflagellates *Gymnodinium catenatum* under four environmental conditions (Tr1: 19 °C and 8.0 pH; Tr2: 24 °C and 8.0 pH; Tr3: 19 °C and 7.6 pH; Tr4: 24 °C and 7.6 pH), showing sampling dates during uptake (grey) and elimination (white) phases.

Table 1
PST content and toxin profile of the *Gymnodinium catenatum* culture used to feed mussels.

Toxin	Concentration (fmol cell^{-1})	Molar fraction (%)
C1 + 2	40.80	95.7
GTX5	1.01	2.4
dcNeo	0.42	1.0
dcGTX2 + 3	0.31	0.7
dcSTX	0.1	0.2
C3 + 4	[a]	[a]
GTX6	[a]	[a]

[a] Not quantified.

cultured in 2 L flasks with seawater adjusted to 30‰ and enriched with GSe medium as in Doblin et al. (1999) without the soil extract. Seawater and nutrients solutions were filtered and autoclaved to minimise contamination. The cultures were grown at 18 °C with a 12:12 L:D cycle under fluorescent lights. Cells were harvested when cultures presented a density of approximately 2.5 × 10^6 cells per litre, concentrated using 10 µm mesh sieve. The toxin profile of the algae was composed by sulfocarbamoyl toxins and decarbamoyl toxins (Table 1). Toxins were determined in the algae cell culture as described below (Section 2.4).

2.3. Mussels exposure to toxic dinoflagellates

During acclimation, mussels were fed with 100,000 cells per day per animal of the non-toxic and freeze-dried, *Tetraselmis* sp. diet (Necton, Olhão, Portugal). The mussels were then fed for 5 days with toxic dinoflagellates from a *G. catenatum* culture under the conditions indicated in the previous section and following the experimental design shown in Fig. 1. Mussels were exposed to approximately 91,000 *G. catenatum* cells per day per animal. After the 5 days of exposure to the toxic diet, mussels were fed again with 100,000 cells per day per animal of *Tetraselmis* sp. during 10 days in order to assess the elimination of toxins accumulated during the 5-days exposure period. Two mussels exposed to *G. catenatum* were collected in triplicate for toxin analysis on days 1 and 5, corresponding to the uptake period, and on days 6, 10 and 15, corresponding to the elimination period.

2.4. Determination of PSTs

2.4.1. Reagents

All reagents used for toxins extraction and analysis were of analytical grade or higher. Acetic acid glacial (100%, p.a.), methanol (> 99.8%, p.a.) and acetonitrile (Analytical grade) were obtained from Sigma-Aldrich; ammonium formate (> 99% purity) from Fluka; Hydrochloric acid (37%) from Panreac; Hydrogen peroxide (30%) and sodium hydroxide (> 99%, p.a.) from Merck. Water was purified using a Milli-Q 185 Plus system from Millipore. Toxins standard solutions for dcGTX2 + 3, C1 + 2, dcSTX, GTX2 + 3, GTX5 and STX, were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council (NRC, Canada).

2.4.2. Extraction and determination of PSTs by liquid chromatography with fluorescence detection

Extraction of toxins from *G. catenatum* cell cultures followed the methodology described by Silva et al. (2015). Briefly, an aliquot of *G. catenatum* cell culture (500 mL) was filtered onto 47 mm Whatman GF/C with a nominal pore size of 1.2 mm, under low vacuum. Toxins were extracted in 4 mL of 0.05 M acetic acid and sonicated for 4 min at 25 W, 50% pulse duty cycle (Vibracell, Sonic & Materials, Newtown, CT, USA) in an ice bath. Cell lysis was confirmed with light microscopy. The extract was then centrifuged (3000*g*) for 10 min, and 1 mL of the supernatant was used for the determination of PSTs. The supernatant was cleaned by solid-phase extraction (SPE) with an octadecyl bonded phase silica (Supelclean LC-18 SPE cartridge, 3 mL, Supelco, USA). Briefly, the SPE cartridge was conditioned with 6 mL of methanol, followed by 6 mL of water and 1 mL of extract was loaded to the cartridge and the PSTs eluted with 2 mL of water.

Both extraction of toxins from mussels and the determination of the PSTs followed the EU reference method (AOAC Official Method 2005.06) which consists on a Liquid chromatographic separation

coupled to fluorescence detection (LC-FLD) with precolumn derivatization, to convert the toxins into the correspondent fluorescent derivatives. Briefly, 5 g of shellfish whole soft tissue homogenate was double extracted with 1% acetic acid solution (first extraction with heating), and the extracts were cleaned using solid-phase SPE C18 cartridge (Supelclean, Supelco, USA). Before LC/FLD analysis, 100 µL of C18 extract from both algae and mussels was derivatized with peroxide and periodate oxidants.

LC-FLD analysis were performed on a Hewlett-Packard/Agilent LC system (Germany) constituted by a Model 1100 quaternary pump, Model 1100 in-line degasser, autosampler, column oven, and Model 1200 fluorescence detector. The Hewlett-Packard Chemstation software performed data acquisition and peak integration. Toxins separation was performed using a reversed-phase column Supelcosil C18 column 150 × 4.6 mm id, 5 µm (Supelco, USA) equipped with a column Supelguard Supelcosil C18, 20 mm id × 4.0 mm id, 5 µm (Supelco, USA) and kept in a column oven at 30 °C. The flow rate was 1 mL min^{-1} and the mobile phase gradient used to elute the PSTs oxidation products consisted of 2 mobile phases under the following conditions: 0–5% B (0.1 M ammonium formate in 5% acetonitrile, pH = 6) in the first 5 min, 5–70% B for the following 4 min and 100% mobile phase A (0.1 M ammonium formate, pH = 6) used during 5 min before the next injection. The injection volume was 50 µL, detection wavelengths were set at 340 nm for excitation and 395 nm for emission. PSTs were identified by comparison with standards retention times and quantified by interpolation in the calibration curves obtained with the factor response peak areas *vs.* toxin concentration (see an illustrative LC-FLD chromatogram of PSTs in Supplementary material Fig. A.2). The limits of detection (signal-to-noise ratio of 3) ranged from 0.004 µM for C1 + 2, GTX5 and STX to 0.020 µM for dcNEO. The toxicity equivalent factors stated by EFSA (2009) were used for calculation of PSTs in terms of saxitoxin equivalents. The total PSP toxicity in the sample corresponds to the sum of all toxin analogues quantified. For toxins co-eluting and determined together (C1 + 2, dcGTX2 + 3, and GTX2 + 3) the higher toxicity equivalent factor of the co-eluted compound was used. Low potency PSTs analogues for which analytical standards were not available, namely C3 + 4 and GTX6, were not considered for this study.

2.4.3. Statistics

Two-Way Analysis of Variance followed by a Multiple Comparisons *versus* Control Group (Bonferroni *t*-test) was used to assess significant differences on toxin content and toxicity between the current conditions, warming, acidification and the combined effect of warming and acidification. Also, a Two-Way Analysis of Variance followed by All Pairwise Multiple Comparison Procedures (Bonferroni *t*-test) was used to assess significant differences between the sampling dates.

For the empirical kinetics of PSTs elimination, a one-compartment model was used to describe elimination kinetics using a single component first-order kinetic model:

$$\frac{dC_m}{dt} = -k_{el} C_m \quad (1)$$

where C_m is the toxin concentration in mussels and k_{el} denotes the elimination rate. Solving this differential equation gives:

$$C_m = C_{m0} e^{-k_{el} t} \quad (2)$$

The toxin concentration decreases according to an exponential decay, with the steepness of the decay being determined by the elimination rate (k) and the size of the curve depending of the initial concentration of the toxin (C_{m0}) at the beginning of the elimination period, when mussels diet was changed from *G. catenatum* to non-toxic algae.

Data were tested for normality and homogeneity of variance by the Kolmogorov-Smirnov test and the Levene Median test. Differences were considered significant at $p < 0.05$. Data analysis was performed using the statistical program SigmaPlot Version 10.0.

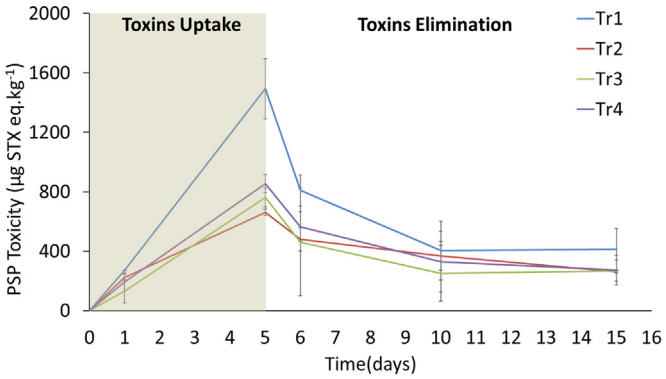

Fig. 2. PSP toxicity (µg STX eq. kg^{-1}, mean ± SD) determined in mussels exposed to toxic *Gymnodinium catenatum* during 5 days and to non-toxic diet during the subsequent 10 days, under four environmental conditions (Tr1: 19 °C and 8.0 pH; Tr2: 24 °C and 8.0 pH; Tr3: 19 °C and 7.6 pH; Tr4: 24 °C and 7.6 pH).

3. Results

3.1. Impact of warming and acidification on mussels toxicity

A marked increase of PSTs accumulation was observed in mussels during the uptake period in all treatments (Fig. 2). The highest toxin levels were determined in mussels at the current conditions of temperature and pH (Tr1: 19 °C and pH 8), which in terms of shellfish toxicity expressed as saxitoxin equivalents after multiplying the toxins concentration with the respective toxicity factor, reached levels of 1493.8 ± 202.4 µg STX eq. kg^{-1} at day 5. PSP toxicity was significantly ($p < 0.001$) lower in acidification- and warming-acclimated mussels, reaching values of 761.2 ± 62.3 and 661.9 ± 22.8 µg STX eq. kg^{-1}, respectively. Although similar to the toxicity observed for treatments 2 and 3, where the effect of each climate change driver was evaluated individually, the combined effect of both components, tested in treatment 4, resulted in PSP toxicity values slightly higher (855.8 ± 61.4 µg STX eq. kg^{-1}).

When algae provided to mussels was changed to non-toxic species, a decrease of toxicity was immediately observed in all treatments. The decrease of PSP toxicity was particularly evident in mussels at current conditions. For these mussels, a significant decrease ($p < 0.001$) was observed after 24 h feeding on non-toxic algae, corresponding to a toxicity reduction of 57.4 ± 3.2%. The highest toxicity decrease in the first 24 h was found in acidification treatment (66.9 ± 5.0%), and the lowest decrease of only 27.3 ± 12.9% was registered for mussels maintained in the warming treatment. The interaction between the two variables conducted to a decrease of 47.6 ± 25.7%. Toxicity reached similar levels for all treatments in the subsequent days of the elimination period (Fig. 2).

3.2. Accumulation and elimination kinetics of PSTs under conditions of warming and acidification

The profile of PSTs detected in mussels, as illustrated in Fig. 3, was constituted by the same toxins found in the toxic algae given as food, namely C1 + 2, GTX5, dcGTX2 + 3, dcSTX and dcNeo.

At the end of the 5-day uptake period, significantly higher concentrations of each PST congener were observed in mussels acclimated at current conditions than in the other treatments. Exception occurred for the concentration of C1 + 2, which was determined at levels similarly high in mussels at current (Tr1) and acidified (Tr3) conditions.

Significant differences in concentration of PSTs were also found between the combined effect of warming and acidification (Tr4) with each component tested individually (Tr2 or Tr3). Indeed, higher C1 + 2 concentrations were determined in Tr4 than in Tr2, but reaching lower

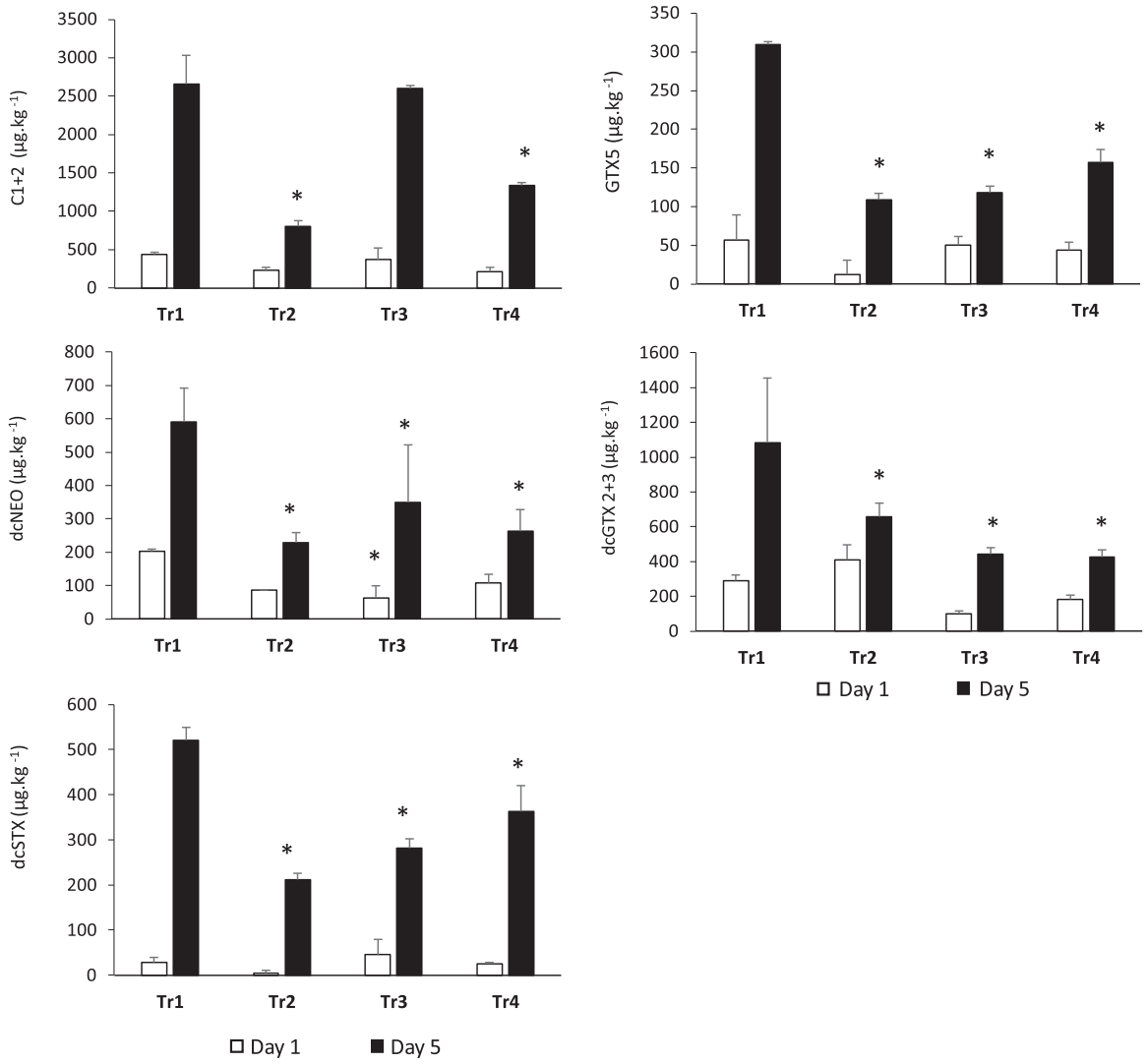

Fig. 3. Concentration (µg kg^{-1}, mean ± SD) of PSTs (C1 + 2, GTX5, dcNeo, dcGTX2 + 3 and dcSTX) determined in mussels exposed to *Gymnodinium catenatum* at day 1 (white bars) and 5 (black bars), under four environmental conditions (Tr1: 19 °C and 8.0 pH; Tr2: 24 °C and 8.0 pH; Tr3: 19 °C and 7.6 pH; Tr4: 24 °C and 7.6 pH). Values marked with an asterisk represent significant differences ($p < 0.05$) from current conditions (*vs.* Tr1) within the same exposure time.

levels than in Tr3. Higher GTX5 and dcSTX concentrations were determined in Tr4 than in Tr2 and Tr3. Finally, concentrations of dcGTX2 + 3 reached lower values than Tr2, but not statistically different to Tr3.

After reaching the peak of toxins accumulation in day 5, the shift to non-toxic diet resulted in a decrease of toxins concentrations. However, toxins were not completely eliminated during the studied period (10 days). The timeline of toxins elimination in each treatment is illustrated in Fig. 4. All toxins fitted well to an exponential decay model, except dcGTX2 + 3. Elimination rates calculated from the best fit are indicated in Table 2. The highest elimination rates were generally obtained for mussels under acidifying conditions, and lower elimination rates were registered in mussels under warming conditions. The combination of both climate change drivers resulted in an attenuation of effects, leading to elimination rates between mussels at current and acidified conditions.

C1 + 2, that were initially the dominant PSTs found in algae and mussels from the four treatments, was easily eliminated in mussels maintained in acidification conditions. On the other hand, these toxins were slowly eliminated from mussels under warming conditions. The lowest elimination rates were calculated for GTX5 in all treatments. The most potent compound from this set of toxins, *i.e.* dcSTX, was rapidly eliminated in mussels under current conditions compared to any other treatment.

4. Discussion

Climate change is a complex phenomenon, resulting from alterations on several environmental drivers. Temperature might be the easiest parameter to follow, but climate changes include several other parameters with direct and indirect impact on pH, salinity, oxygen levels, current regimes and even frequency and intensity of harmful algal blooms (HAB). Therefore, it is essential to understand the interaction of these variables, which may lead to either antagonistic or synergistic responses of marine organisms (Duarte et al., 2014; Lischka et al., 2011).

HAB-toxins represent a significant threat to commercial shellfish farming, frequently leading to closures to harvesting due to toxins accumulation at levels above the international regulatory limits. In this study, mussels feeding during 5 days on PSTs-producing dinoflagellate *Gymnodinium catenatum* exceeded the EU regulatory limit of 800 µg STX eq. kg^{-1} when maintained under the current conditions of temperature and pH and in the combined acidification and warming conditions. In contrast, mussels acclimated solely to warming and acidification displayed significantly lower PSP toxicity levels, *i.e.* below but close to the safety limits. Seafood demand has grown in the last decades and is

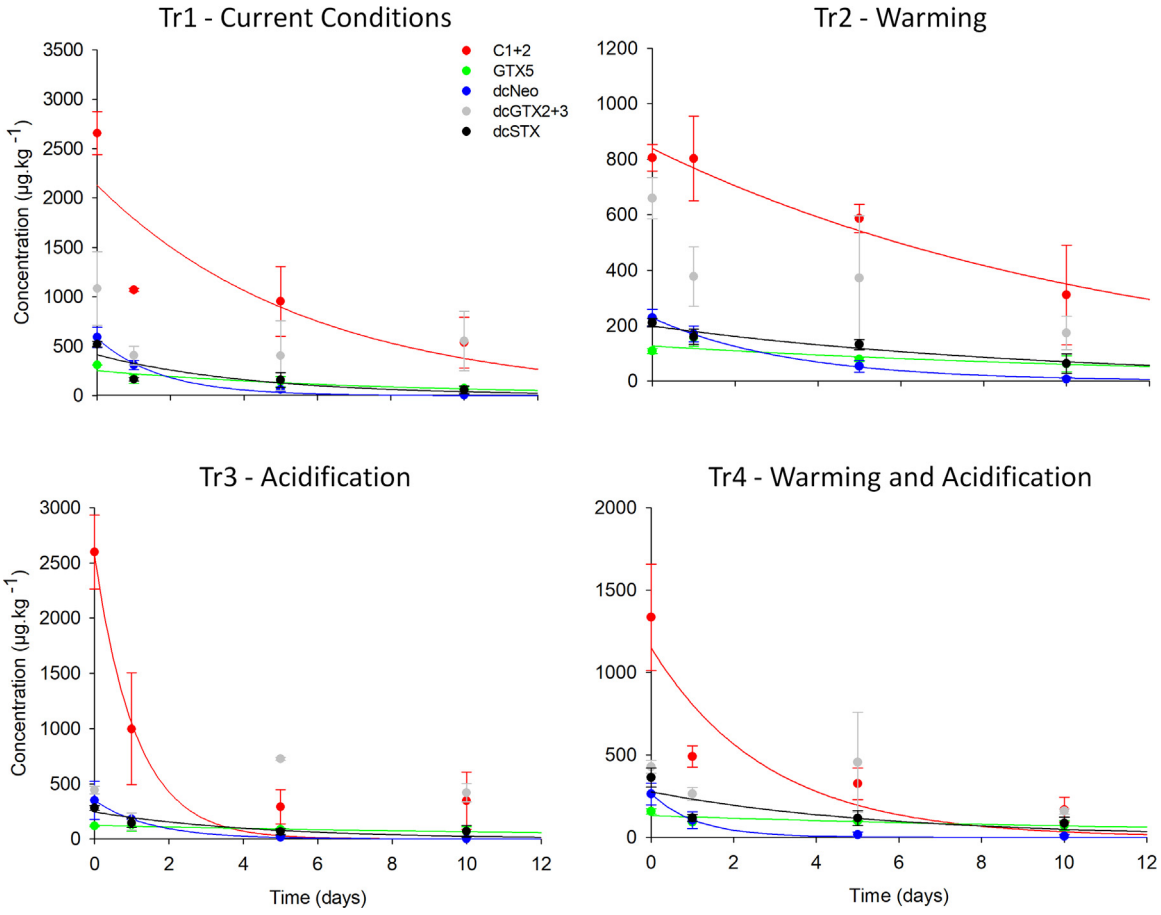

Fig. 4. Elimination of the PSTs (C1 + 2, dcSTX, GTX5, dcNeo and dcGTX2 + 3) in mussels under four environmental conditions (Tr1: 19 °C and 8.0 pH; Tr2: 24 °C and 8.0 pH; Tr3: 19 °C and 7.6 pH; Tr4: 24 °C and 7.6 pH). Dots and error bars represent experimental data; the coloured lines represent the output of the simulation model ($C_m = C_{m0}\, e^{-kel\ t}$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Elimination rate (k_{el}, d^{-1}) (± standard error) and coefficient of determination R^2 of each PST determined in mussels under four environmental conditions (Tr1: 19 °C and 8.0 pH; Tr2: 24 °C and 8.0 pH; Tr3: 19 °C and 7.6 pH; Tr4: 24 °C and 7.6 pH).

Treatment	Toxin	Elimination rate		R^2
Tr1 Current Conditions	C1 + 2	0.173	(± 0.052)*	0.81
	GTX5	0.131	(± 0.035)*	0.82
	dcNeo	0.578	(± 0.076)*	0.98
	dcSTX	0.234	(± 0.071)*	0.82
Tr2 Warming	C1 + 2	0.087	(± 0.016)*	0.89
	GTX5	0.074	(± 0.024)*	0.73
	dcNeo	0.300	(± 0.037)*	0.98
	dcSTX	0.105	(± 0.018)*	0.90
Tr3 Acidification	C1 + 2	0.898	(± 0.219)*	0.93
	GTX5	0.063	(± 0.027)*	0.63
	dcNeo	0.658	(± 0.243)*	0.90
	dcSTX	0.231	(± 0.064)*	0.85
Tr4 Warming and Acidification	C1 + 2	0.355	(± 0.114)*	0.86
	GTX5	0.064	(± 0.021)*	0.71
	dcNeo	0.904	(± 0.194)*	0.95
	dcSTX	0.174	(± 0.062)*	0.73

* Represents values within the confidence limit ($P < 0.05$).

expected to increase at a higher level with the global population growth. For this reason, sustainable increased production, *i.e.* aquaculture, is seen as the solution to meet the demand for shellfish that cannot be supported by the declining wild stocks. According to PSP toxicity results in this study, one could suggest that climate change, regarding warming and acidification, may benefit shellfisheries as lower toxicity levels are reached. Nevertheless, the decrease of toxicity during the elimination period was significantly slower in acclimated mussels than in mussels maintained at current environmental conditions. Assuming that HAB are increasing in frequency and intensity (Fu et al., 2012) and that toxin-producing dinoflagellates under acidified conditions are prone to increase their toxicity (Tatters et al., 2013), shellfish are expected to become more frequently contaminated. Moreover, shellfish may remain contaminated for longer periods, although not reaching the actual exacerbated levels.

Most studies on climate change effects in shellfish have focused on a single driver (*e.g.* temperature, acidification, salinity) (Anestis et al., 2010, 2007; Farrell et al., 2015; Fernández-Reiriz et al., 2012). A reduced number of studies addressed the combination of two variables. In this study, the individual impact of each parameter was tested to better understand the effects of their interaction. Analyzing warming as a single climate change driver in mussels exposed to toxic algae lead to a reduction of toxicity levels. The same trend was previously described by Farrell et al. (2015) in one of the few studies combining climate change drivers, namely increased temperature and biotoxin accumulation. A reduction in both accumulation and elimination of the PSTs GTX1 + 4 and GTX2 + 3 were observed in warming-acclimated oysters fed with toxic *Alexandrium minutum*. Reduction of oysters PSP toxicity under warming conditions was not concurrent with decrease of clearance rates and routine metabolism (Farrell et al. 2015). In contrast, a reduction of nearly 50% of clearance rates were reported in mussels *M. galloprovincialis* under warming conditions (Anestis et al., 2010). This reduction was suggested to be related with a metabolic depression mechanism in order to balance the increase in energy demand due to warmer water temperatures (Anestis et al., 2010). Although clearance

and metabolic rates were not assessed in the present study, lower PSP toxicity values in warming-acclimated mussels may result from changes on these physiological components.

In addition to increasing temperature, the effect of acidification was also investigated. Ocean acidification is not plausible without temperature rising. However, to better understand the role of this climate change driver, mussels were acclimated to an acidification scenario with and without warming. Testing this parameter individually also allowed to investigate the hypothesis that lower pH conditions may promote the elimination of PSTs. This hypothesis was raised in 1965, but not positively tested (Hayes, 1966; Shumway et al., 1995). Suspecting that pH could affect shellfish physiology and favor toxins elimination, Hayes (1966) submitted PST contaminated butter clams (*Saxidomus giganteus*) to extreme acidified pH values (5 and 6) for 15 h at 7 °C without promising results. In the present study, significant decrease in mussel toxicity was observed, reclaiming this hypothesis as a way to promote PSTs elimination.

In the present study, the lower toxicity levels found for *M. gallo-provicialis* under acidified conditions appear to be related with different mechanisms than those associated with toxicity reduction at increasing temperatures. *M. galloprovicialis* acclimated to seawater acidification conditions (*i.e.* to pH decrease of 0.3 or 0.6 units) have shown to maintain their clearance, ingestion and metabolic rates (Fernández-Reiriz et al., 2012). Alternatively, some digestive enzymes may present higher activities (Areekijseree et al., 2004; Fernández-Reiriz et al., 2012). Indeed, induction of enzymatic activities and antioxidants was found at a pH around 7.3 and 7.7 (Hu et al., 2015). In *M. coruscus*, an increase in the reduced glutathione (GSH) consumption was assessed under acidification conditions (Hu et al., 2015). The consumption of this antioxidant is particularly relevant since GSH has been found to have an important role on biotransformation and elimination of PSTs analogues (Costa et al., 2012; Sakamoto et al., 2000; Sato et al., 2000). Assessing the variability of each PST analogue throughout the experiment was essential to better understand the individual effects of warming and acidification. Although both climate change drivers resulted in lower PSP toxicity levels, the individual effects of warming and acidification differed on the accumulation and elimination of several PST analogues determined.

The *N*-sulfocarbamoyl analogues C1 + 2 were the most abundant toxin congeners found in mussels during the uptake period in all treatments, as they were the most abundant PST identified in the microalgae. However, C1 + 2 concentration was significantly higher in mussels maintained under acidification conditions than in warming-acclimated mussels. In fact, the concentration of C1 + 2 was similar to mussels under current conditions, suggesting high uptake rates and no effects on clearance rates, thus supporting previous findings mentioned above (*i.e.* Fernández-Reiriz et al., 2012).

The C1 + 2 are the least potent of the PST derivatives, making variations in their concentrations less relevant for total PSP toxicity. On the other hand, variability of decarbamoyl toxin analogues, in particular, dcSTX that is the most potent compound of this set of toxins, notably impacts mussels PSP toxicity. The accumulation of these analogues was identically reduced in mussels maintained at either warming or acidification, which was the main factor for decreasing toxicity levels in mussels subjected to climate change drivers compared to mussels at current conditions.

The effects of warming and acidification also differed during the elimination period. The experimental data concerning the concentration of toxins determined in mussels during this period fitted well to the dynamic model, providing a good description of the kinetics of C1 + 2, GTX5, dcNeo and dcSTX in mussels. C1 + 2 that dominated mussels profile in all treatments are the less stable (Kodama, 2010) and easier eliminated PST derivatives (Botelho et al., 2010; Li et al., 2005; Lopes et al., 2014; Yu et al., 2007). The highest C1 + 2 elimination rates were calculated in mussels under acidification conditions (Tr3), which highly accumulated these toxins during the uptake period. Mussels of this treatment (Tr3) revealed similar or slightly higher elimination rates of dcSTX and dcNeo than mussels maintained at current environmental conditions. The lowest elimination rate in mussels at Tr3 was calculated for GTX5. Reduced elimination rates of GTX5 were also obtained for warming-acclimated mussels and under the combined effect of warming and acidification. These elimination rates support the theory discussed before referring acidification as a possible way to promote toxins elimination. Despite no clear biotransformation pattern was detected in our study, an enhancement of biotransformation enzymes described under acidified conditions (Hu et al., 2015) could be behind these rates. Warming-acclimated mussels presented the lowest or nearly the lowest elimination rates in all toxin congeners, pointing out a decrease in mussels metabolic activity as suggested by Anestis et al. (2010).

The effects of both climate change drivers, *i.e.* warming and acidification, lead to a reduction of PSP toxicity, but mussels subjected to the combined effect of both drivers showed only slightly higher toxicity levels than mussels subjected solely to warming or acidification. This fact highlights the need for more studies approaching the exposure to multiple climate change drivers.

5. Conclusion

The present work is the first study assessing PSTs accumulation and elimination in mussels under the combined effects of warming and acidifying environments, indicating that such climate change scenarios may lead to lower but prolonged PST contamination in *Mytilus galloprovincialis*. Although the combined effects of warming and acidification promoted lower PSP toxicity levels than the current conditions, the underlying kinetics differed among them. While warming-acclimated mussels accumulated low toxin levels and may retain them due to low elimination rates, mussels at acidification conditions showed higher ability to accumulate PSTs, but their high elimination rates prevented mussels to reach high toxicity levels as mussels maintained under current conditions.

This study also highlights an approach with potential interest for shellfisheries industry affected by *Gymnodinium catenatum* blooms, as lower seawater pH may promote elimination of PSTs. Although further studies are needed to confirm this hypothesis and to set the optimal parameters to promote such elimination without conditioning shellfish shell growth and well-being in future depuration facilities.

Acknowledgements

This study was supported by CERES project (European Union's Seventh Framework Programme – FP7 /2007–2013). Ana C. Braga has a Doctoral Grant (PD/BD/113484/2015) from the Portuguese Science and Technology Foundation (FCT). António Marques and Pedro R. Costa are supported through the FCT Investigator program (IF). This work contributes to project UID/Multi/04326/2013 from the Portuguese Foundation for Science and Technology (FCT). Thanks are due for the financial support to CESAM (UID/AMB/50017 – POCI-01-0145-FEDER-007638), to FCT/MCTES through national funds (PIDDAC), and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2018.03.045.

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